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Short Communication

Immobilization of heparin on polyacrylamide derivatives

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Abstract

Heparin was coupled via its carboxyl group with a polyacrylamide derivative containing covalently bound amino groups using the carbodiimide reaction. Heparin immobilized in this way proved to be useful as an affinity carrier for the isolation of antithrombin III and heparin-binding proteins from boar seminal plasma.

1. Introduction

Polyacrylamide-based affinity carriers are useful in the affinity chromatography of various proteins. Their easy preparation makes them suitable matrices for the binding of a chosen ligand with the desired degree of substitution. They can be prepared in water-insoluble form as matrices for affinity chromatography [1], or in water-soluble form as the macromolecular derivative of a ligand for the study of protein–ligand interactions [2–4].

The approach chosen in this work for binding heparin to polyacrylamide copolymers was by coupling heparin via its free carboxyl group to amino groups of a polyacrylamide derivative. Heparin, owing to its unique structure and surface charge distribution, is able to interact strongly with several different proteins. This paper describes a method of heparin immobilization and the application of the prepared gel as an affinity carrier for the isolation of two different kinds of proteins interacting with this ligand.

2. Experimental

2.1. Materials

Boar semen was collected from healthy Large White boars using an artificial vagina in the Center for the Development of Insemination (Rajhrad u Brna, Czech Republic). Boar seminal plasma was isolated from sperm by centrifugation at 600 *g* for 20 min at 5°C. Freshly frozen human plasma was obtained from the Blood Transfusion Service, heparin from Léčiva (Prague, Czech Republic) and affinity purified antithrombin III from the Institute of Sera and Vaccines (Prague, Czech Republic). 1-(3-Dimethyl-aminopropyl)-3-ethylcarbodiimide was purchased from Fluka (Buchs, Switzerland) and heparin-Sepharose from Pharmacia (Uppsala, Sweden).

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2.2. Preparation of water-insoluble polyacrylamide copolymers containing amino groups

A polyacrylamide derivative containing covalently bound amino groups was prepared by copolymerization of acrylamide and allyl amine in the presence of a cross-linking agent. Acrylamide (3 g) and allylamine (1.2 ml) were dissolved in 45 ml of distilled water, then the pH of the solution was adjusted to 7.0 using 6 *M* HCl. After dilution to 60 ml, $(NH_4)_2S_2O_8$ (190 mg) was added. The solution was heated to 95°C on a water-bath and then kept at 95°C for 30 min. After being cooled, the gel formed was disintegrated by pushing it through the nylon net and exhaustively washed with distilled water.

2.3. Coupling of heparin to a polyacrylamide derivative containing free amino groups

The carbodiimide reaction was chosen to couple heparin via its carboxyl groups to amino groups of the prepared polyacrylamide derivative. Solutions of heparin (500 mg in 5 ml of water) and of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide (200 mg in 5 ml of water) were added to the prepared gel suspended in 170 ml of distilled water. The suspension was shaken for 24 h at room temperature and then washed with distilled water.

The amount of immobilized heparin, determined either by the method described by Smith *et al.* [5] or by the phenol–sulphuric acid method [6], was in the range 1.2–1.5 mg/ml or 200–220 μg/mg. For long-term storage, the suspension of the prepared gel can be lyophilized without a change in binding capacity.

2.4. Isolation of heparin-binding proteins from boar seminal plasma

Boar seminal plasma (25 ml) diluted with phosphate-buffered saline (PBS) (25 ml) was applied to a column (28 × 3.2 cm I.D.) of immobilized heparin to polyacrylamide pre-equilibrated with PBS. The non-adsorbed proteins were washed with PBS and the adsorbed proteins were eluted with 3 *M* NaCl in PBS. Fractions of 7.8 ml per 5 min were collected. The course of the chromatography was followed by the measurement of the absorbance at 280 nm. Fractions containing adsorbed proteins were pooled, dialysed against 0.2% acetic acid and then lyophilized.

The same procedure was used for affinity chromatography on heparin-Sepharose [7].

2.5. Isolation of antithrombin III

Affinity chromatography was performed as described by Smith *et al.* [8]. Human plasma (500 ml) after the removal of cryoprecipitate and filtration (Seitz filter, EKS, diameter 30 cm) was applied to a column of immobilized heparin (10 ml) and washed with 0.02 *M* phosphate buffer (pH 7.3) containing 0.15 *M* NaCl. The column was then washed with 0.02 *M* phosphate buffer (pH 7.3) containing 0.35 *M* NaCl (60 ml). The bound antithrombin III was eluted with 0.02 *M* phosphate buffer (pH 7.3) containing 2 *M* NaCl. The eluted antithrombin III was dialysed exhaustively against distilled water and then lyophilized. The course of affinity chromatography was followed by the measurement of absorbance at 280 nm and by the determination of the antithrombin III activity [9].

The capacity of immobilized heparin was determined using affinity-purified antithrombin III; the conditions of affinity chromatography were the same as described above.

2.6. Electrophoresis in the presence of SDS

Polyacrylamide gel electrophoresis in the presence of SDS was performed in 12.5% gels according to the routine procedure [10]. Proteins from boar seminal plasma isolated by affinity chromatography were dissolved in non-reducing buffer (2 μg per 1 μl of buffer) and heated for 10 min at 60°C. Bovine serum albumin, carbonic anhydrase, ribonuclease and cytochrome *c* were used as standards. After electrophoresis the gel was stained with Coomassie Brilliant Blue R.

3. Results

3.1. *Isolation of heparin-binding proteins from boar seminal plasma*

The prepared affinity carrier was used for the isolation of heparin-binding proteins from boar seminal plasma. The course of affinity chromatography on heparin immobilized on polyacrylamide was very similar to that on heparin-Sepharose (Fig. 1). Proteins isolated by both methods show identical bands when subjected to the electrophoresis in the presence of SDS (Fig. 2).

3.2. *Isolation of antithrombin III*

Heparin immobilized on the polyacrylamide derivative was used for the isolation of antithrombin III from human plasma. The results of affinity chromatography are summarized in the Table 1. The yield of antithrombin III from 500 ml of blood plasma using a 10-ml column was 10–12 mg of lyophilized product with an activity of 4.8–1.7 I.U./mg (chromatography was repeated three times).

The electrophoretic behaviour of the obtained preparation of antithrombin III in polyacrylamide electrophoresis in the presence of SDS was comparable to that of the antithrombin III standard. The average capacity of the immobilized heparin on polyacrylamide gel was 1.4 mg of antithrombin III per 1 ml of the gel with an activity of 5.6 I.U./mg.

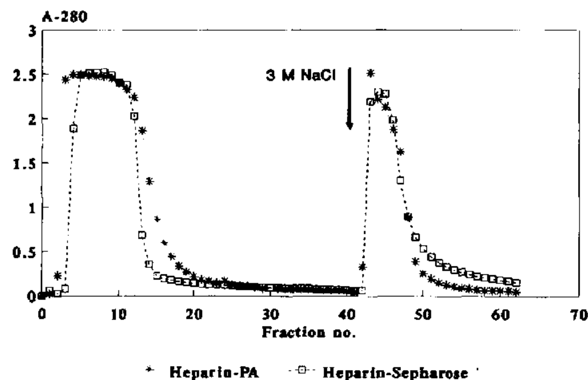

Fig. 1. Affinity chromatography of boar seminal plasma on heparin immobilized (*) on polyacrylamide derivative (PA) and (□) on Sepharose.

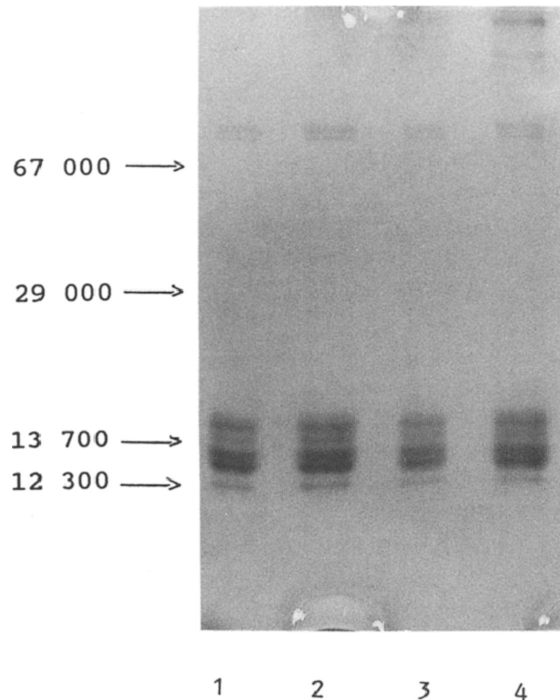

Fig. 2. Affinity purified heparin-binding proteins from boar seminal plasma after polyacrylamide gel electrophoresis in the presence of SDS: 1 and 2, 10 or 20 μg of protein after affinity chromatography on heparin bound to polyacrylamide derivative; 3 and 4, 10 or 20 μg of protein after affinity chromatography on heparin-Sepharose.

Table 1
Affinity chromatography of human plasma on immobilized heparin

Protein fraction	Antithrombin III activity (I.U./ml)
Human plasma applied to the column	0.90
Fraction I eluted with starting buffer	0.76
Fraction II (antithrombin III)	4.7

4. Discussion

Heparin is an unbranched, highly sulphated glycosaminoglycan; when immobilized on an insoluble beaded polymer it can be used for the purification of many proteins and biomolecules [11–15]. Usually heparin is attached to the matrix via its hydroxyl groups. In our procedure, we used an alternative approach, choosing its free carboxyl group for attachment to the polyacrylamide derivative. The amide bond formed is stable; no leakage of the ligand was observed in repeated experiments. The solid matrix used for the heparin immobilization represents a new type of carrier, which could also be used for the immobilization of other ligands containing not only carboxyl groups, but also, for example, aldehyde groups; the latter case might be useful for the immobilization of ligands containing saccharide moieties (after periodate oxidation).

A further advantage of the described method is the possibility of preparing a water-soluble macromolecular derivative of heparin; this method might be suitable for solid-phase studies of the protein–heparin interactions [16], similarly as was shown for O-glycosyl–polyacrylamide copolymers [4].

5. References

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